



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12Q 1/02, C12N 5/00, C07D 207/00, 209/00, 295/00	A1	(11) International Publication Number: WO 00/34507 (43) International Publication Date: 15 June 2000 (15.06.00)
(21) International Application Number: PCT/US99/28769 (22) International Filing Date: 7 December 1999 (07.12.99) (30) Priority Data: 60/111,195 7 December 1998 (07.12.98) US (71) Applicant (for all designated States except US): DUKE UNIVERSITY [US/US]; 230 North Building, P.O. Box 90083, Durham, NC 27708-0083 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): SMITH, Clayton, A. [US/US]; Duke University, Hematology & Medical Oncology, Dept. of Medicine, 421 Medical Science Research Building, Durham, NC 27710 (US). COLVIN, Michael [US/US]; 208 Arcadia Lane, Chapel Hill, NC 27514 (US). STORMS, Robert, W. [US/US]; 30 Dansey Circle, Durham, NC 27713 (US). LUDEMAN, Susan, M. [US/US]; 7313 208 Calibre Park Drive, Durham, NC 27707 (US). (74) Agent: WILSON, Mary, J.; Nixon & Vanderhye P.C., Suite 800, 1100 North Glebe Road, Arlington, VA 22201-4714 (US).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: A METHOD OF ISOLATING STEM CELLS (57) Abstract The present invention relates, in general, to stem cells, and in particular, to a method of isolating stem cells and to reagents suitable for use in such a method. The invention further relates to stem cell populations isolatable in accordance with the present method.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

A METHOD OF ISOLATING STEM CELLS

TECHNICAL FIELD

The present invention relates, in general, to stem cells, and in particular, to a method of isolating stem
5 cells and to reagents suitable for use in such a method. The invention further relates to stem cell populations isolatable in accordance with the present method.

BACKGROUND

10 The most primitive hematopoietic stem cells (HSC) will reconstitute all of the hematopoietic lineages for an entire lifespan. These pluripotent hematopoietic stem cells (PHSC) are the transplantable cells that are ultimately the targets for gene delivery in stem cell-
15 based gene therapies. One defining characteristic for PHSC is that they will survive most cytoablative conditioning regimens. The mechanisms for their resistance to these toxic agents suggest potential strategies by which these cells can be selected in
20 vitro. One mechanism for drug resistance lies in the ability to efflux toxic substances out of the cell via the multiple drug resistance (MDR) pump. Fluorescent substrates for the MDR pump have permitted the isolation of PHSC based on their high capacity for dye
25 efflux in a variety of assay systems. Drug resistances may also be conferred by more specific mechanisms. For

example, a cytosolic aldehyde dehydrogenase (ALDH) mediates resistance to cyclophosphamide (CPA), an alkylating agent used in cytoreductive regimens in preparation for bone marrow transplant. Thus,
5 expression of ALDH can be considered a selectable marker for true PHSC.

The therapeutic effectiveness of CPA has been attributed largely to the ability of PHSC and intestinal crypt cells to survive the drug regimen.
10 Human hematopoietic progenitors express a cytosolic ALDH and primitive human HSC derived from mobilized peripheral blood stem cells can be selected when placed in culture with cyclophosphamide for 7 days. Jones et al have demonstrated that long-term reconstituting
15 murine PHSC can be isolated by providing a membrane-permeable fluorescent substrate for ALDH and by then selecting cells with the highest levels of ALDH activity (Jones, Blood 85:2742 (1995); Jones et al, Blood 88:487 (1996)). In these studies, dansyl
20 aminoacetaldehyde (DAAA) was used to stain murine bone marrow cells prepared by countercurrent elutriation.

Preliminary studies using DAAA indicate that this reagent is unusable on preparations of human hematopoietic cells because the signal intensity of the
25 reagent is too high to resolve discrete cell populations by flow cytometry. The present invention provides a fluorescent ALDH substrate that is free of the problems associated with DAAA and that can be used in the purification of primitive human hematopoietic
30 cells.

SUMMARY OF THE INVENTION

The present invention relates to a novel reagent and method for isolating stem cells, including human stem cells. The reagent is a fluorescent substrate for
5 ALDH. The method comprises staining a cell population that includes primitive stem cells with the substrate in the presence of an inhibitor of MDR activity. ALDH present in the cells converts the substrate to a product that is trapped within the cells. Since
10 primitive stem cells have higher levels of ALDH activity than other cell types, these cells stain brighter than other cell types. The presence of the MDR inhibitor reduces the efflux of the substrate from the stem cells.

15 Objects and advantages of the present invention will be clear from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1D. BAAA staining identifies cells with high levels of ALDH activity. L1210/cpa is a
20 derivative of the L1210 leukemic cell line that overexpresses ALDH. (Figs. 1A and 1B represent L1210 cells, plus DEAB and minus DEAB, respectively; Figs. 1C and 1D represent L1210/cpa cells, plus and minus DEAB, respectively).

25 Figures 2A-2D. BAAA is effluxed by an MDR pump from hematopoietic cells, particularly primitive CD34+ cells, as evidenced by the difference between the CD34+

cells that are BODIPY^{bright} (Fig. 2B) in the presence and absence of the MDR inhibitor, verapamil (Figs. 2A and 2B are at t=0, minus and plus verapamil, respectively; Figs. 2C and 2D are at t=30', minus and plus verapamil, respectively.)

Figures 3A-3D. ALDH^{br} cells (i.e., cells with low SSC properties that stain brightly with BAAA in the presence of an MDR inhibitor) are enriched for cells with the primitive CD34⁺CD38^{lo/-} immunophenotype traditionally associated with primitive stem cells.

Figures 4A and 4B. The staining intensity with BAAA correlates inversely with CD38 (Fig. 4A) and CD71 (Fig. 4B) expression in CD34⁺ cells.

Figures 5A-5C. ALDH^{br} cells are enriched for early progenitors equivalent to CD34⁺ cells and are more enriched for very primitive progenitors than CD34⁺ cells. (Fig. 5A = progenitors (HPCA), Fig. 5B = early progenitors (5 week LTC) and Fig. 5C = primitive progenitors (8 week LTC)).

Figure 6. Preparation of BAAA. Using an amber vial, a solution of aminoacetaldehyde diethyl acetal (0.019 mmol, Aldrich Chemical Co.) in dry tetrahydrofuran (THF, 0.5 mL) was added dropwise to a solution of BODIPY FL, SE (0.013 mmol, Molecular Probes) in dry THF (0.5 mL). Upon complete addition, the vial was capped and the reaction mixture was stirred for 30 min. The THF was evaporated and the residue was dissolved in minimal methylene chloride and

then chromatographed on silica gel using ethyl acetate - hexane (1:1) as eluent. The product, BODIPY-aminoacetaldehyde diethylacetal (BAAA) was recovered in quantitative yield and identified by proton NMR.

5

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method of isolating stem cells and to a reagent suitable for use in such a method. The method comprises contacting a population of cells comprising stem cells with a
10 detectable substrate for aldehyde dehydrogenase (ALDH), which substrate is converted to a detectable product by ALDH, that product being retained in the cells. In a preferred embodiment, the substrate is BODIPY-aminoacetaldehyde (BAAA) and efflux of BAAA from the
15 cells, particularly the stem cells present in the population, is inhibited by the concurrent use of a MDR-inhibitor.

Sources of cell populations that are suitable for use in the present invention include umbilical cord
20 blood, bone marrow, peripheral blood and fetal liver. Any cell population that includes stem cells can be used regardless of tissue origin (e.g., gut, skin muscle, nerve, etc.). While the present method can be expected to be applicable to a variety of non-human
25 mammalian cell populations, it is particularly useful in isolating human stem cells from sources including those referenced above.

Substrates suitable for use in the present invention include substrates for ALDH, particularly specific substrates for ALDH, that are detectable or bear a detectable label and that are converted, by the action of ALDH to products that are detectable or bear the detectable label which products are retained in the cells, particularly, the stem cells. In a preferred embodiment, the substrate is a fluorescent substrate that has a discrete fluorescence emission profile identical to FITC. An example of such a substrate is BAAA.

The optimum amount of substrate to be added to the cell population can be readily be determined by one skilled in the art (see Example). In the case of BAAA, concentrations can vary, for example, concentrations of about 1 μ M to 5 μ M can be used.

A concentrated solution of the substrate can be added directly to medium comprising the cells to be stained or harvested cells can be suspended in a substrate-containing medium.

In order to inhibit efflux of the substrate of the invention from the cells, concurrent use of an inhibitor of MDR is preferred. Any of a variety of MDR inhibitors can be used, including verapamil. The inhibitor can be added to the cells simultaneously with the substrate or prior to the addition of the substrate. The optimum amount of MDR-inhibitor to be used can be readily determined (e.g., by monitoring loss of staining). In the case of verapamil,

concentrations can vary, for example, a concentration of about 50 μ M can be used.

After exposure of the cell population to the substrate (and the MDR inhibitor) (e.g., about
5 30 minutes after), those cells that contain higher concentrations of labeled product can be separated from those that contain lower concentrations. In the case of the use of a fluorescent label, fluorescence
activated cell sorting techniques can be used. Stem
10 cells can be purified from other cells of the starting population based on low orthogonal light scattering on a flow cytometer (identifies small cells, like lymphocytes) and/or brightness of fluorescence. As shown in the Example that follows, sorting the
15 brightest 1% of cells yielded a nearly 40-fold enrichment for cells that initiate long term cultures. The cell preparations that were recovered up to 65% CD34⁺ cells, most of which were CD38⁻/_{dim} CD71^{-dim}. The invention includes within its scope cell preparations
20 that are greater than 50% CD34⁺ cells, preferably greater than 75% CD34⁺ cells, more preferably, greater than 90% CD34⁺ cells.

The stem cells isolated in accordance with the present invention have application in a variety of
25 therapies and diagnostic regimens. They are suitable for both transplantation and gene therapy purposes. For example, isolation of stem cells from bone marrow or peripheral blood of patients with cancer can provide for the separation of stem cells from cancer cells. In
30 patients undergoing autologous transplantation, such

separation can be used to reduce the chance that cancer cells are returned to the patient. Purified autologous stem cells can be ex vivo expanded to hasten neutrophil, erythroid and platelet engraftment after autologous transplantation. Ex vivo expansion can be effected by growth in defined cytokines, on stromal layers and/or in bioreactors (Emerson et al, Blood 87:3082 (1996)). In addition, the incidence of graft failure can be reduced. This is beneficial for cancer patients undergoing autologous transplantation, for patients suffering from auto-immune disorders, and for patients undergoing gene therapy.

Gene therapy approaches involving the present cells involve, in one embodiment, isolation of autologous stem cells, exposure of the isolated cells to a gene delivery vector and re-infusion of the modified cells into the patient (Smith, J. Hematother. 1:155 (1992)). This approach can involve ex vivo culture or the use of vectors capable of transferring genes into non-dividing cells, thereby rendering ex vivo culture unnecessary. Gene therapy can be useful in treating, for example, congenital diseases, such as sickle cell anemia, in which case the mutant β -globin gene is replaced or supplemented with either the wild type globin gene or an anti-sickling globin gene. In the treatment of cancer, drug resistance genes can be introduced into the stem cells to confer resistance to cytotoxic drugs. This can reduce the incidence and severity of myelosuppression. For the treatment of

infectious diseases, including HIV, anti-viral genes can be introduced into the stem cells so that they are rendered resistant to the virus (Gilboa and Smith, Trends in Genetics 10:139 (1994)).

5 Isolation of stem cells results in the elimination of T-cells that cause GvHD. This elimination can be expected to reduce the incidence and severity of GvHD in recipients of allogeneic transplants.

Purified allogeneic stem cells can be ex vivo
10 expanded to hasten neutrophil, erythroid and platelet engraftment after allogeneic transplantation. In addition, the incidence of graft failure can be reduced. This is likely to be particularly important for recipients of umbilical cord blood transplants
15 where small cell doses limit the success of transplantation.

Successful engraftment with stem cells can also be expected to induce tolerance. Such would clearly enhance solid organ transplantation.

20 It will be appreciated that cells of the present invention can be used as sources of new genes (eg for cytokines and cytokine receptors), including genes important in growth and development.

In addition to their application in treatment and
25 diagnosis strategies, the stem cells of the invention can be used in screening protocols to identify agents that can be used, for example, to promote differentiation or growth and/or engraftment of hematopoietic cells. In one such protocol, stem cells
30 are contacted with a test compound suspected of

inducing differentiation and the ability of the test compound to effect differentiation determined (using, for example, microscopic and flow cytometric examination). In another screening protocol, stem
5 cells are contacted with a test compound suspected of inducing proliferation and/or engraftment and the ability of the test compound to effect proliferation and/or engraftment determined using *in vitro* long term colony assays or *in vivo* immunodeficient mice models
10 (eg SCID NOD mice). (See Peault et al, Leukemia 7:s98-101 (1993)).

In addition to the above, the substrate of the invention can be used to identify tumors that may be resistant to cyclophosphamide via up regulation of ALDH
15 activity. In accordance with this embodiment, cells of the tumor can be contacted with the detectable substrate, e.g., BAAA, and MDR inhibitor under conditions such that the substrate enters the cells and is converted therein to the detectable product. Cells
20 that stain brightly (e.g., with BAAA) can be expected to be cyclophosphamide resistant.

The invention also relates to kits that can be used to prepare the cells of the invention. The kits can comprise reagents (e.g., ALDH substrate) that can
25 be used to effect isolation of the stem cells. In a preferred embodiment, the kit includes BAAA disposed within a container means. The kit can also include, disposed within a container means, an MDR inhibitor, such as verapamil.

Certain aspects of the present invention are described in greater detail in the non-limiting Examples that follow.

EXAMPLE 1

5 Experimental Procedures

Preparation of BODIPY aminoacetaldehyde.

The aldehyde dehydrogenase substrate is prepared as BODIPY aminoacetal and lyophilized in 0.5 micromole aliquots. These preparations are stable indefinitely
10 when stored at -20°C. The acetal is then solubilized in DMSO to a final concentration of 5 mM. This solution has been found to be stable at 4°C for up to 1 week. To convert the acetal to an acetaldehyde, aliquots of this solution are brought to a final
15 concentration of 1 N HCl. Under these conditions the acetal has a half life of 15 minutes. After 2 hours in 1 N HCl, the vast majority of the BODIPY aminoacetal has converted to BODIPY aminoacetaldehyde (BAAA), and is then diluted to 200-250 mM in Dulbecco's phosphate
20 buffered saline (PBS). This stock is added directly to cells prepared in Iscove's Modified Dulbecco's Medium (IMDM) with 2% FCS at concentrations ranging from 1 to 5 μ M.) (See also Fig. 6.).

Antibody reagents.

25 Directly-conjugated fluorescent antibodies directed against CD2 (Leu5; FITC), CD3 (Leu4; PerCP), CD5 (Leu1; PE), CD7 (Leu9; FITC), CD10 (CALLA; FITC), CD11b (Leu15; PE),

CD14 (Leu M3; PE), CD19 (Leu12; FITC), CD33 (LeuM9; PE), CD34 (HPCA2; FITC and PE), CD38 (Leu17; PE), CD56 (Leu19; PE) and HLA-DR (FITC) from Becton Dickinson Immunocytometry Systems (BDIS; San Jose, CA) were used. Anti-CD7 (3A1; PE) and anti-CD45 (KC56; PE) were purchased from Coulter Corporation (Hialeah, FL); anti-CD3 (UCHT1; PE), anti-CD16 (3G8; PE), anti-CD19 (J4.119; PE) as well as the pooled anti-CD34 antibodies (QBEnd10, Immu-133, Immu-134; PE) from Immunotech, Inc. (Westbrook, ME); anti-CD3 (B-B11; FITC) and CD38 (B-A6; FITC) from BioSource International (Camarillo, CA); anti-CD45RA (F8-11-13; PE) from Southern Biotechnology Associates, Inc. (Birmingham, AL); and anti-CDw90 (5E10; PE) from PharMingen, Inc. (San Diego, CA).

Cell lines.

K562, L1210 and L1210/cpa cells (ATCC) were maintained in suspension in RPMI 1640 media supplemented with 10% Fetal Calf Serum (FCS) and 5×10^{-5} M β -mercaptoethanol.

Preparation of Human Umbilical Cord Blood.

Human umbilical cord blood (UCB), intended for disposal, was collected into sterile bottles containing anticoagulant citrate buffer. The UCB used in these studies were processed within 24 hours of being harvested. White cells were enriched through a preliminary red cell agglutination where the UCB was diluted 1:2 with Dulbecco's phosphate buffered saline (PBS) at room temperature. These cells were then brought to a final concentration of 1% Hespan (DuPont Pharma, Wilmington, DE) and were left to stand undisturbed for 1 hour. Non-agglutinated white blood

cells were harvested and residual red cells were hemolysed at 37°C in 0.17 M NH₄Cl containing 10 mM Tris-HCl, pH 7.2 and 200 mM EDTA. The recovered cells were washed in IMDM containing 2% FCS and mononuclear cells are then purified using Ficoll-Hypaque (1.077 g/ml). When held overnight, the cells were kept on ice in a 4°C refrigerated room in IMDM with 20% FCS.

Cell staining and fluorescence-activated cell sorting.

Mononuclear UCB cells were resuspended at 10⁶ cells/ml in IMDM containing 2% FCS and were labeled with 1 µM BAAA for 30 min. When used, verapamil was included at 50 mM. After staining, the cells were washed with ice cold staining media and maintained on ice until their analysis and sorting. The cells were then resuspended in staining media with 10 mg/ml 7-aminoactinomycin D (7AAD) (Molecular Probes; Eugene, OR). For antibody staining to permit multiparameter analyses, the cells were resuspended in staining media (100 µl) and antibodies were added directly to the cell suspensions. The cells were incubated on ice for 20 min. and then washed again in ice cold staining media. The cells were then analyzed or sorted on a FACStar Plus cell sorter (BDIS) equipped with dual Coherent I-90 lasers + an argon-dye laser. The BAAA was excited at 488 nm and emissions were detected using 515 DF20 filter in FL1. Dead and dying cells were excluded on the basis of their high emission in the far red wavelength due to their uptake of 7AAD.

For analyses of cell surface antigens on cells previously sorted based on BAAA staining, the cells were

pelleted and resuspended in IMDM with 2% FCS. The cells were then held at 37°C for 1-2 hours to permit efflux. The cells were then pelleted and fluorescence-conjugated antibodies were added directly to the cells. Following incubations for 20 minutes, the cells were washed with PBS/2% FCS and were fixed in 1% formaldehyde in PBS/2% FCS. In all surface marker analyses, no differences were noted between analyses with cells stained simultaneously with BAAA and with antibodies and those analyses performed on FACS® sorted cells that were subsequently stained with antibodies.

Hematopoietic progenitor colony assays and long term cultures.

ALDH^{br} cells were isolated directly from mononuclear UCB cells which had been stained with BAAA. For these assays, the ALDH^{br} was defined as 1% of the lymphocyte gate of the UCB.

Hematopoietic progenitor colony assays were performed by plating 100-200 cells in MethoCult H4431 containing agar leukocyte conditioned media and recombinant human erythropoietin (StemCell Technologies, Inc.). The cells were incubated in a humidified chamber at 37°C with 5% CO₂. Hematopoietic colonies (>100 cells) were then scored at 14 to 18 days after initiating the cultures. Long term cultures were maintained on stromal layers of murine MS-5 cells (provided by Dr. Tadashi Sudo of the Kirin Pharmaceutical Research Laboratory, Gunma, Japan) (Issaad, Blood 81:2916 (1993)). MS-5 stromal cells were seeded into 24-well plates (Corning Costar Corp., Cambridge, MA) at 5 X

10⁴ cells/well in DMEM supplemented with 10% FCS and cultured at 37°C. When the monolayers approached 80% confluence they were γ-irradiated from a cesium source (40 Gy). After irradiation, fresh media was provided to the cultures. For the MS-5 cells, the culture media was replaced entirely with MEMα supplemented with 10% FCS, 10% equine serum, β-mercaptoethanol, pyruvate. Long term cultures were initiated with 400-2000 hematopoietic progenitor cells/well and were maintained at 33°C with 5% CO₂. At weekly intervals half the media from each well was removed so that the media could be replenished. Adherent and non-adherent cells were harvested after 5 or 8 weeks and plated into HPC assays as described above. As shown in the Example that follows, sorting the brightest 1% of cells yields a nearly 40-fold enrichment for cells that initiate long term cultures (how can higher levels of enrichment be achieved or would cell cloning be used at this point??). The cell preparations that were recovered were up to 65% CD34⁺ cells, most of which were CD34⁺ cells, most of which were CD38^{-dim} CD71^{-dim}.

Results

Synthesis of BODIPY acetal.

Due to the inherent instability of aldehydes in aqueous solution, the reagent is prepared and stored as an acetal. Immediately prior to its use, the acetal is converted to an aldehyde in 1 N HCL. the aldehyde is freely soluble in PBS and can be added directly to cells prepared in IMDM with 2% fetal calf serum at 10⁶

cells per ml. As an aminoacetaldehyde, the reagent is membrane permeable; however, in the presence of the aldehyde dehydrogenase (ALDH), the aldehyde moiety is converted to a carboxylic acid that is retained in the cell. Intracellular fluorescence can be used to select cells.

BAAA is a Specific Substrate for ALDH.

To assay whether BAAA would permit the specific selection of ALDH⁺ cells, studies initially determined an optimal response dose for the BAAA reagent in a murine cell line previously selected for cylophosphamide-resistance, L1210/cpa, that is known to be ALDH⁺ (Fig. 1). The parental cell line, L1210 (Figs. 1A and 1B) is cylophosphamide-sensitive and ALDH⁻. This cell line exhibited essentially no response to BAAA. In addition, a potent inhibitor of ALDH, diethylbenzaldehyde (DEAB), was used to demonstrate the specificity of the BAAA signal. A 10-fold molar excess of DEAB totally blocked the fluorescent response (Fig. 1C). Therefore, BAAA was able to detect ALDH⁺ cells. In these studies, the BAAA could be used at a final concentration as low as 5 μ M. This molar concentration is 10-fold lower than that used with the dansylated reagent.

Multiple different ALDH isoenzymes exist and these may display different abilities to convert BAAA. It has been suggested that resistance to cyclophosphamide is primarily mediated by a specific ALDH isoenzyme, ALDH1. Therefore, a human cell line known to express

ALDH1, K562, was assayed with this novel reagent. K562 cells converted BAAA and were positive for ALDH in these assays. This response was entirely inhibited by DEAB. Thus, BAAA can serve as a specific substrate for human ALDH1 and can be used to identify primary human cells that demonstrate resistance to cyclophosphamide.

Primary UCB Cell preparations contain subsets of ALDH^{br} cells.

Having demonstrated the effectiveness of this reagent on continuous cell lines, BAAA was assayed on primary human cells. Umbilical Cord Blood (UCB) was chosen for its increasing promise as a source for transplantable hematopoietic stem cells. For these studies, the UCB was unfractionated except for having been prepared for mononuclear cells over Ficoll-Hypaque. This separation is significant in that two mature ALDH⁺ cell types, erythrocytes and megakaryocytes, are removed. The BAAA was tested on UCB cells prepared in IMDM with 2% FCS at 10^6 cells/ml (Fig. 2). The UCB cells were very responsive to the BODIPY reagent, and appeared to be much more sensitive than the continuous cell lines had been. The BAAA was therefore titrated to an optimal concentration of $1\mu\text{M}$. This was the best concentration for resolving ALDH^{br} subpopulations. The response was inhibited in the presence of excess DEAB and was therefore specific for ALDH. This molar concentration is 50-fold lower than the concentration of dansyl aminoacetaldehyde that had

previously been used to detect murine pluripotent hematopoietic stem cells.

The fluorescence emission from BAAA-stained UCB cells exhibited a bimodal response. The brighter peak of fluorescence emission was attributed to mature monocytes, suggesting monocytes express a uniform level of ALDH. Hematopoietic stem cells are small, non-complex cells. Indeed, murine ALDH⁺ PHSC were first enriched using countercurrent elutriation. Therefore, the BODIPY signal was examined only in non-complex cells that exhibited low inherent orthogonal light scattering (SSC^{lo}) (Fig. 3A). The majority of the SSC^{lo} UCB cells were ALDH^{neg/dim} (Fig. 1B). This was not unexpected since the SSC^{lo} cells are predominantly lymphocytes, and most lymphocytes do not express ALDH. However, a small, clearly-defined subpopulation of the SSC^{lo} UCB cells was ALDH^{br} (Fig. 3A).

BODIPY aminoacetate is a substrate for the MDR efflux Pump.

In addition to expressing ALDH, PHSC should also express high levels of the P-glycoprotein or multiple drug resistance (MDR) efflux pump. Since this reagent had never been previously characterized, the susceptibility of BAAA to MDR efflux was assayed. Although BODIPY aminoacetaldehyde passes through the cell membrane without active transport, the product of the ALDH conversion (BODIPY aminoacetate) might well be a substrate for the MDR pump. To investigate this possibility, UCB cells were stained with BAAA in the

presence of 50 μ M verapamil, a competitive inhibitor of the MDR efflux pump. The verapamil-treated cells exhibited a consistently-higher fluorescence when compared with BAAA-stained cells that had not been simultaneously treated with verapamil (Fig. 2). A substantial population of ALDH^{dim} cells were effected by the verapamil treatment. Most importantly, the percentage of ALDH^{br} cells increased by 1.8 fold in the presence of verapamil. In verapamil-treated cells, the ALDH^{br} subpopulation was equivalent to 0.8 \pm % of the SSC^{lo} cells. In contrast, in cell preparations that received no verapamil, the same fluorescence intensity represented only 0.46 \pm % of the SSC^{lo} cells. This indicated that the ALDH^{br} SSC^{lo} UCB cells retain the converted BAAA more effectively if the efflux activity of the MDR pump is inhibited.

ALDH^{br} SSC^{lo} UCB cells are highly enriched for primitive CD34⁺ cells.

With verapamil treatment, the ALDH^{br} SSC^{lo} UCB cells contained almost 90% CD34⁺ cells, indicating that at least some hematopoietic progenitors are present (Fig. 3D). However, CD34 is expressed by a broad range of hematopoietic progenitors that includes lineage committed cells, as well as pluripotent progenitors. Therefore, the developmental potential of the ALDH^{br} SSC^{lo} UCB cells was analyzed. Initially, the immunophenotype of these cells was more carefully defined. The immunophenotype would in no way be conclusive; however, the primitiveness of the cell

population could be inferred by examining 2 activation markers that are typically associated with the differentiation of primitive cells to more lineage-committee hematopoietic cells, CD38 and CD71. The most primitive subsets of CD34⁺ cells have little to no expression of the activation antigens CD38 or CD71. In UCB cell preparations with BAAA and with antibodies specific for CD34 and CD38, the ALDH^{br} SSC^{lo} UCB cells provided a single-step enrichment for essentially purified CD34^{br} CD38^{dim} cells. Furthermore, when CD34⁺ UCB cells were examined independently, ALDH expression was inversely proportional to the expression of both CD38 and CD71 (Fig. 4A and 4B). Thus, the ALDH^{br} SSC^{lo} UCB cells appear to contain the primitive CD34⁺ cells as defined by immunophenotype.

To assay the developmental potential of the ALDH^{br} SSC^{lo} UCB cells, these cells were isolated and placed into both short-term and long-term assays for myeloerythroid progenitors. The short-term assay used, the hematopoietic progenitor colony assay (HPCA), quantifies lineage committed cells at the time of the initial isolation. More primitive progenitors were also assayed by maintaining the ALDH^{br} SSC^{lo} UCB cells on stroma for either 5 or 8 weeks prior to performing the HPCA (Fig. 5).

Results:

HPCA - ALDH^{br} SSC^{lo} essentially equivalent to CD34⁺ cells.

LTC - 5 wk - ALDH^{br} SSC^{lo} essentially equivalent to CD34⁺ cells.

LTC = 8 wk - ALDH^{br} SSC^{lo} outperforms CD34⁺ cells.

EXAMPLE 2

5 ALDH^{br} UCB cells have been shown to be predominately CD34⁺CD38^{-/lo} and highly enriched for early myeloid progenitors. The current study was undertaken to determine whether the ALDH^{br} CD34⁺ UCB cells were enriched for lymphoid progenitors as well.
10 In 3 experiments, cultures of ALDH^{br} CD34⁺ UCB cells were established on AFT024 stromal cells in the presence on Kit ligand, Flt3 ligand, IL-3 (1st day only), IL-2 and IL-7 at various dilutions. After 7-8 weeks, the cultures were analyzed for lymphocyte growth
15 as determined by expression of CD56, CD10, CD19 or CD20.

Table 1			
ALDH ^{br} cells/well	total wells initiated	wells with viable cells	lymphocyte ⁺ wells
1000	6	5	5
250	16	12	12
62	48	40	40
16	48	34	34
10	24	20	17

The AFT024 cultures primarily favored the growth
20 of presumptive NK cells, so to more effectively test whether ALDH^{br} CD34⁺ UCB cells contained B-lymphoid progenitors, they were cultured on the W20 stromal cell

line supplemented with the same cytokine combination.
Of 12 cultures established with 100 ALDH^{br} CD34⁺ cells,
all produced CD56⁺ and CD10⁺ cells at nearly equivalent
proportions. 2 of the 12 wells also contained CD19⁺
5 cells.

In summary, the ALDH^{br} CD34⁺ UCB population appears
to be highly enriched for both myeloid and lymphoid
hematopoietic progenitors.

* * *

10 All documents cited above are hereby incorporated
in their entirety by reference.

One skilled in the art will appreciate from a
reading of this disclosure that various changes in form
and detail can be made without departing from the true
15 scope of the invention.

WHAT IS CLAIMED IS:

1. A method for detecting the presence of stem cells in a population of cells comprising contacting said population of cells with a detectable substrate for aldehyde dehydrogenase (ALDH) under conditions such that ALDH present in cells of said population converts said substrate to a detectable product that is retained by said cells present in said population, wherein said stem cells have a higher concentration of said detectable product than non-stem cells present in said population.

2. The method according to claim 1 wherein said population of cells is a human population of cells.

3. The method according to claim 1 wherein said population of cells is derived from umbilical cord blood, bone marrow, peripheral blood or fetal liver.

4. The method according to claim 1 wherein said substrate bears a fluorescent label.

5. The method according to claim 1 wherein said substrate is BODIPY-aminoacetaldehyde (BAAA).

6. The method according to claim 1 wherein efflux of said substrate from cells of said population

is inhibited by exposing said population of cells to a multiple drug resistance (MDR) inhibitor.

7. The method according to claim 6 wherein said MDR inhibitor is verapamil.

8. The method according to claim 6 wherein said MDR inhibitor is added to cells of said population simultaneously with or prior to addition of said substrate.

9. BODIPY aminoacetaldehyde (BAAA).

10. A composition comprising BAAA and a carrier or diluent.

11. A kit comprising BAAA disposed within a container means.

12. A method of detecting cells present in a population of cells that comprise an elevated level of ALDH comprising contacting said population of cells with a substrate for ALDH bearing a detectable label that is converted to a detectable product that is retained by cells of said population and determining which of said cells of said population have an elevated concentration of detectable product relative to other cells of said population, said cells of said population

having said elevated concentration of detectable product being said cells having an elevated level of ALDH.

13. The method according to claim 12 wherein said cells of said population having an elevated level of ALDH are tumor cells.

14. A method of isolating stem cells present in a population of cells comprising contacting said population of cells with a detectable substrate for aldehyde dehydrogenase (ALDH) under conditions such that ALDH present in cells of said population converts said substrate to a detectable product that is retained by said cells present in said population, wherein said stem cells have a higher concentration of said detectable product than non-stem cells present in said population, and separating said stem cells from said non-stem cells of said population on the basis of said higher concentration of detectable product.

15. The method according to claim 14 wherein said population of cells is a human population of cells.

16. The method according to claim 14 wherein said substrate bears a fluorescent label.

17. The method according to claim 14 wherein said substrate is BODIPY-aminoacetaldehyde (BAAA).

18. The method according to claim 14 wherein efflux of said substrate from said stem cells is inhibited by exposing said population of cells to a multiple drug resistance (MDR) inhibitor.

19. The method according to claim 18 wherein said MDR inhibitor is verapamil.

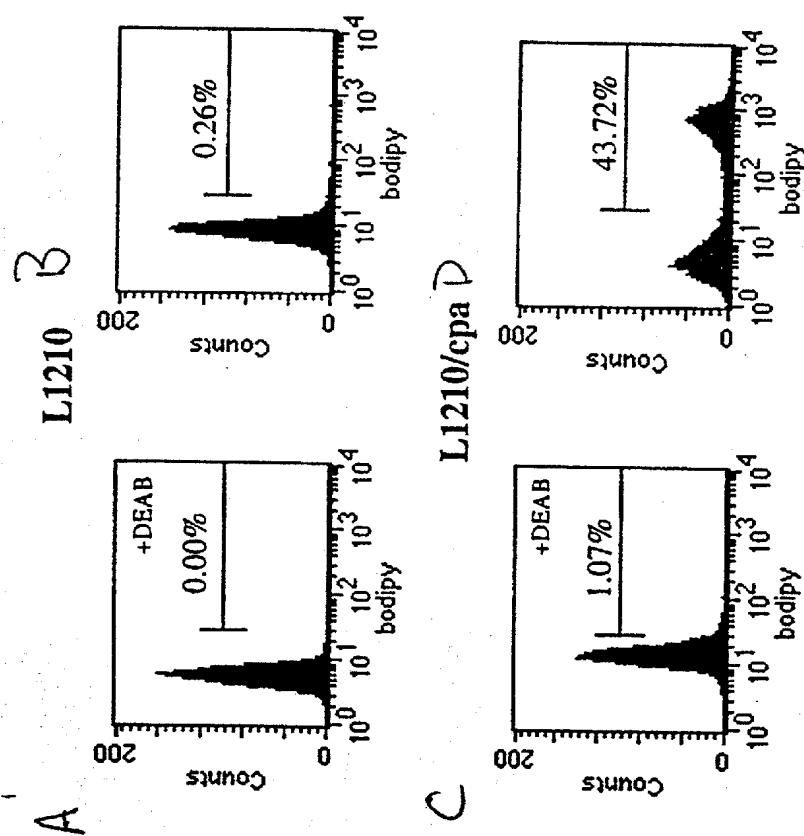


Figure 1. BAAA staining identifies cells with high levels of ALDH activity. L1210/cpa is a derivative of the L1210 leukemic cell line which overexpresses ALDH.

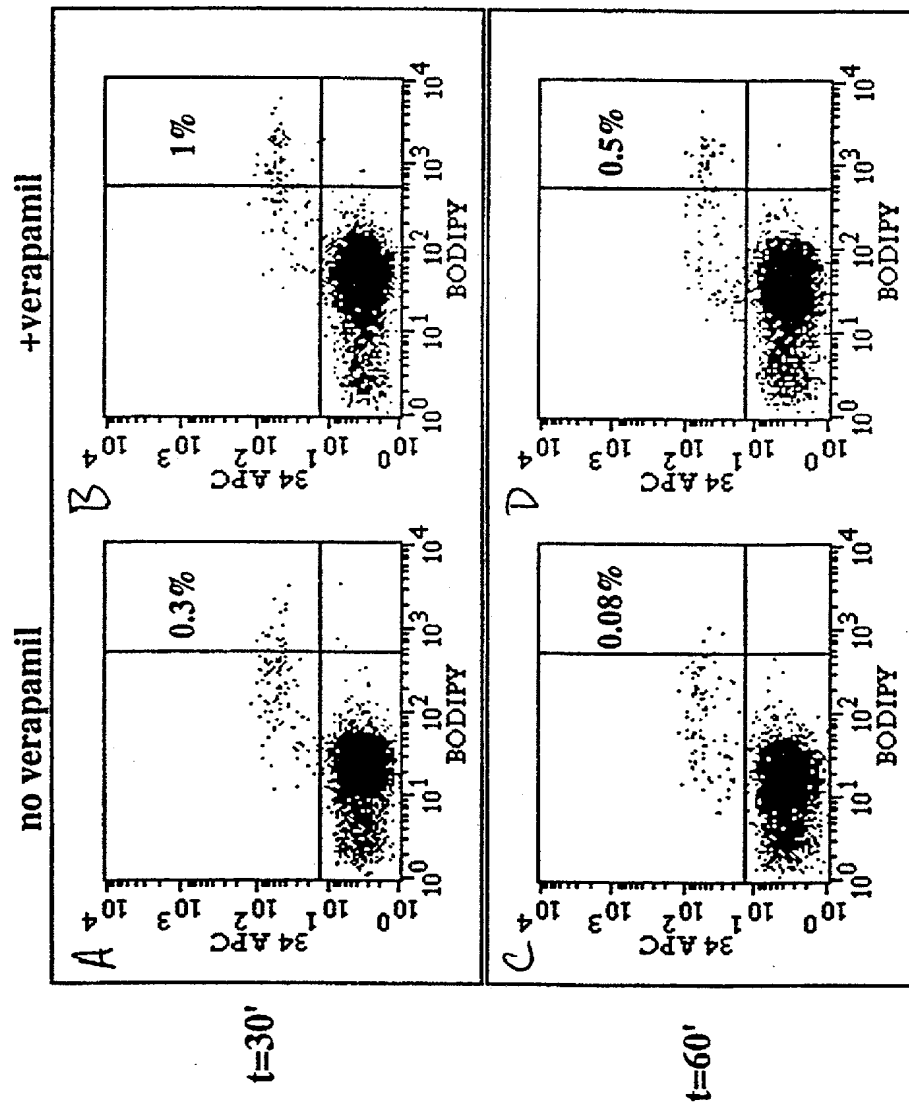


Figure 2. BAAA efflux is blocked by verapamil, an inhibitor of mdr.

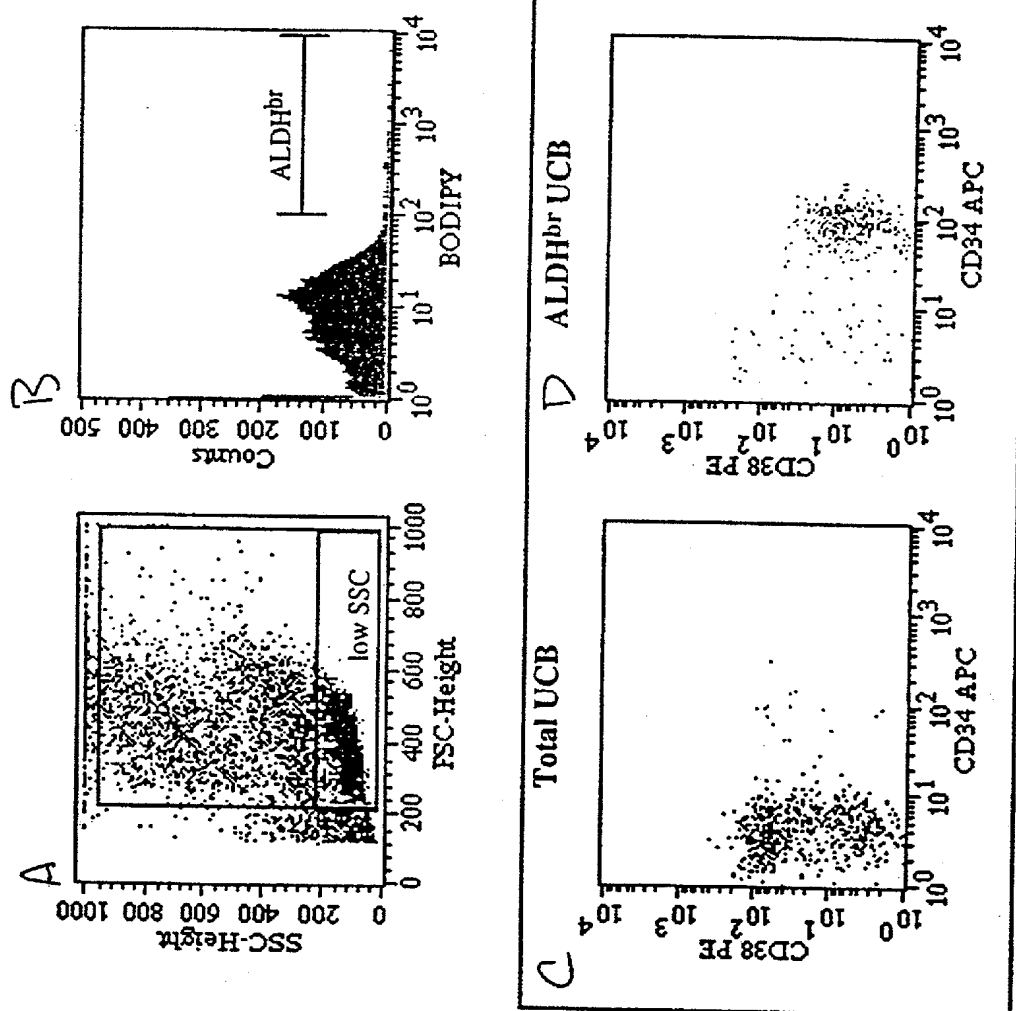


Figure 3. ALDH^{br} cells (i.e. cells with low SSC properties which stain brightly with BAAA in the presence of an mdr inhibitor) are enriched for cells with the primitive CD34⁺CD38^{lo/-} immunophenotype.

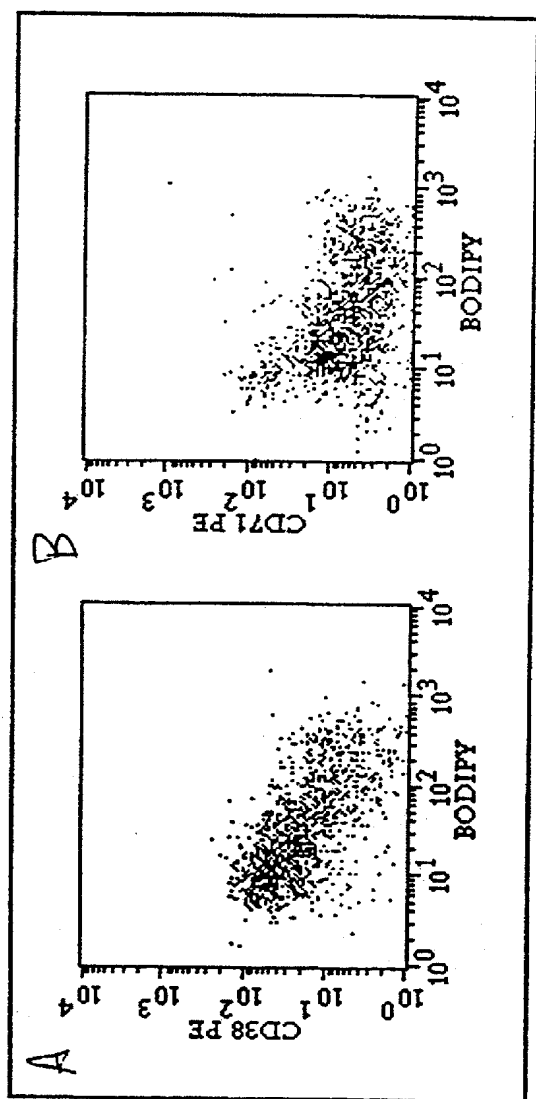


Figure 4. The staining intensity with BAAA correlates inversely with CD38 and CD71 expression in CD34+ cells.

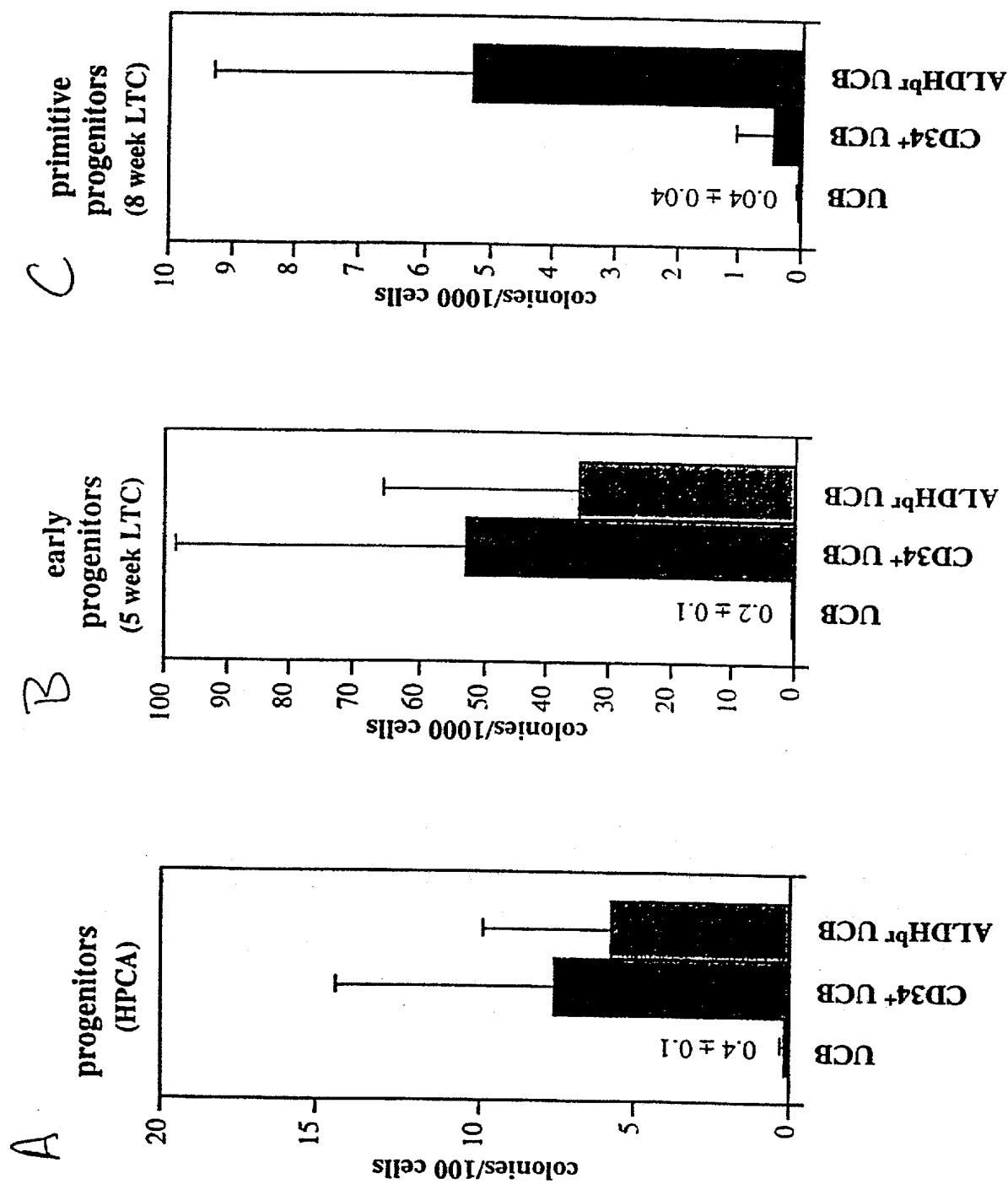
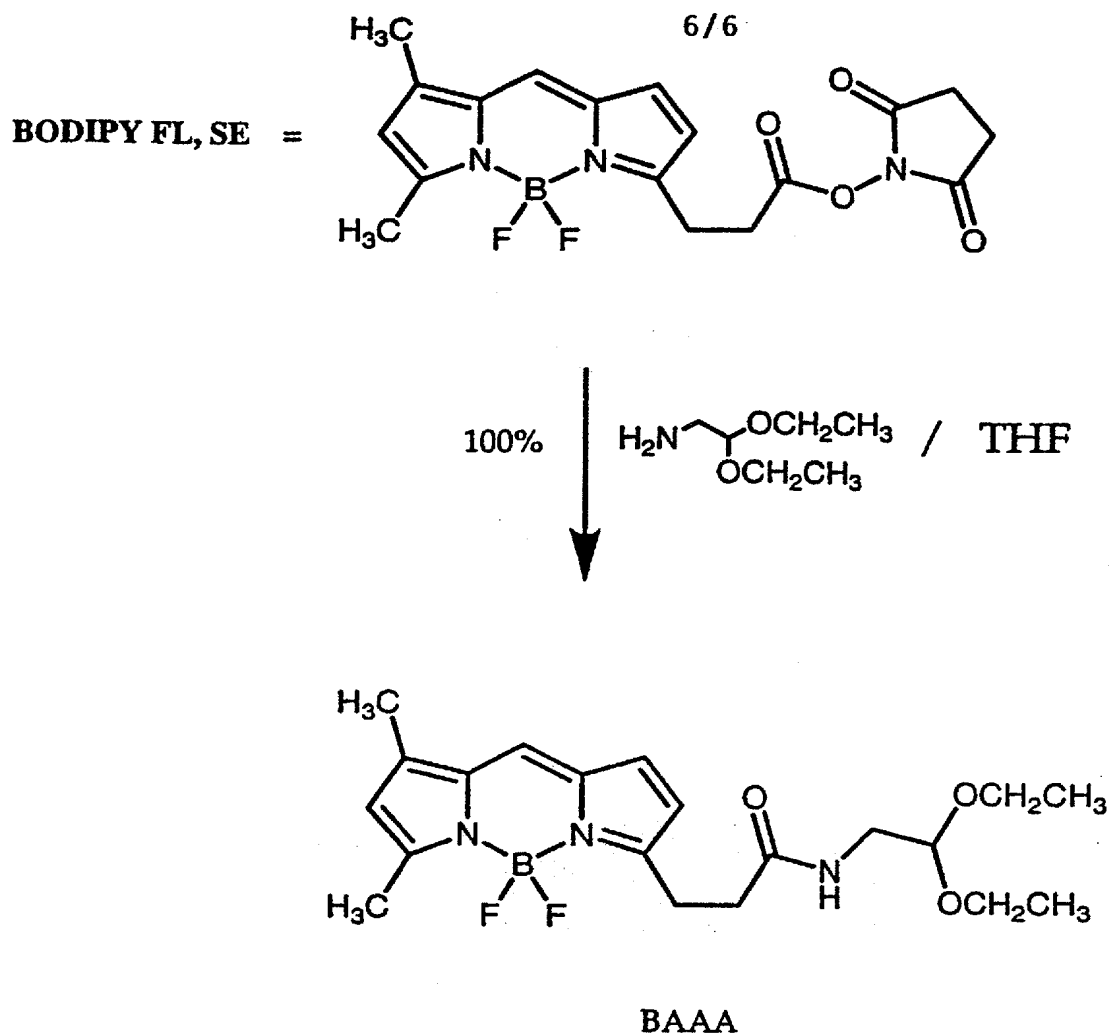


Figure 5. ALDH^{br} cells are enriched for early progenitors equivalent to CD34⁺ cells and are more enriched for very primitive progenitors than CD34⁺ cells.



Using an amber vial, a solution of aminoacetaldehyde diethyl acetal (0.019 mmol, Aldrich Chemical Co.) in dry tetrahydrofuran (THF, 0.5 mL) was added dropwise to a solution of BODIPY FL, SE (0.013 mmol, Molecular Probes) in dry THF (0.5 mL). Upon complete addition, the vial was capped and the reaction mixture was stirred for 30 min. The THF was evaporated and the residue was dissolved in minimal methylene chloride and then chromatographed on silica gel using ethyl acetate - hexane (1:1) as eluent. The product, BODIPY-aminoacetaldehyde diethyl acetal (BAAA) was recovered in quantitative yield and identified by proton NMR.

Fig. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/28769

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12Q 1/02; C12N 5/00; C07D 207/00, 209/00, 295/00
US CL : 435/29, 325, 366; 548/405

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/29, 325, 366; 548/405

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST USPT, DWPI; STN CA, MEDLINE, BIOSIS

search terms: stem cells, aldehyde dehydrogenase, fluorescent aldehyde, BODIPY

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	JONES et al. Assessment of aldehyde dehydrogenase in viable cells. Blood. May 1995, Vol.85, No.10, pages 2742-2746.	1-4,12, 14-16 ----- 5-11,13, 17-19
X ----- Y	JONES et al. Characterization of mouse lymphohematopoietic stem cells lacking spleen colony-forming activity. Blood. July 1996, Vol.88, No.2, pages 487-491.	1,3,4, 12-14,16 ----- 5-11,15, 17-19
Y	LINDAHL, R. Aldehyde dehydrogenase in 2-acetamidofluorene-induced rat hepatomas. Biochemical Journal. 1977. Vol.164, No.1, pages 119-123.	3

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

Special categories of cited documents:			
"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"B"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

02 MARCH 2000

Date of mailing of the international search report

21 MAR 2000

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

VERA AFREMOVA

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/28769

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GIAI et al. Chemoresistance in breast tumors. Eur. J. Gynaecol. Oncol. 1991, Vol.12, No.5, pages 359-374.	6-8,13, 17-19
Y,P	KAISER et al. Determination of the depth of BODIPY probes in model membranes by parallax analysis of fluorescence quenching. Biochimica et Biophysica Acta. 1998, Vol.1375, pages 13-22.	5,9-11,17
X	STORMS et al. Aldehyde dehydrogenase activity as a selectable phenotype for hematopoietic progenitors. Blood. 15 November 1998. Vol.92, No.10 SUPPL. 1 Part 1-2, page 39A.	1-19

Form PCT/ISA/210 (continuation of second sheet)(July 1992)★